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Transient Expression of Dp140, a Product of the Duchenne Muscular Dystrophy Locus, during Kidney Tubulogenesis

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Dystroglycan is a cell surface complex which in muscle links the extracellular matrix protein laminin-2 to the membrane associated cytoskeletal protein dystrophin. Recently it was found that dystroglycan is also expressed in developing epithelial cells. Moreover, antibodies against dystroglycan can perturb epithelial cell development in kidney organ culture. Dystroglycan could provide a link between the basement membrane and the intracellular space also in epithelial cells. However, there is no dystrophin in epithelial cells. By *in situ* hybridization here we show prominent expression of a shorter isoform of dystrophin, Dp140, in embryonic kidney tubules. In addition, another isoform, Dp71, is expressed by all studied embryonic epithelial cells. Both isoforms share the dystroglycan-binding region of dystrophin but lack the region known to bind to actin. Here we also characterized monoclonal antibodies against different domains of dystrophin and used these to study the distribution of Dp140 protein. In embryonic kidney tubules the dystrophin antibody VIA4$_2$A3 stained an intracellular antigen close to the basal cells. In contrast, no staining was observed in adult kidney. We suggest that Dp140 is a structural component during kidney tubulogenesis but it may also be involved in signal transduction.

INTRODUCTION

Basement membrane components and their cell surface receptors are involved in the development of epithelial cells. Antibody perturbation experiments have suggested a role for laminin-1 for epithelial cell development (Klein *et al.*, 1988; Schuger *et al.*, 1991; Ekblom *et al.*, 1994). Laminin-1 is a basement membrane glycoprotein composed of three large polypeptides, the α1, β1, and γ1 chains (Burgeson *et al.*, 1994). Several biologically active domains of laminin-1 have been identified, including the proteolytic fragments E3 and E8. Domain E3 is the carboxy terminus of the α1 chain and E8 is an adjacent larger fragment of the carboxy terminus including all three chains (Timpl and Brown, 1994). The E8 fragment is known to bind to $\alpha 6\beta 1$ integrin (Sonnenberg *et al.*, 1990) and antibody perturbation experiments have suggested that this interaction is important for the development of epithelial cells of the kidney, at least *in vitro* (Sorokin *et al.*, 1990). However, mice lacking integrin α6 subunit undergo normal embryogenesis (Georges-Labouesse *et al.*, 1996), suggesting the presence of many laminin receptors on developing epithelial cells.

Dystroglycan was initially discovered as a laminin-2 receptor in muscle (Ervasti and Campbell, 1993). Our recent studies suggest that dystroglycan also could act as a receptor for laminins during kidney tubulogenesis (Durbeej *et al.*, 1995). Laminins seem to bind to α-dystroglycan via the carboxy-terminal end, which for laminin-1 is the E3 fragment (Ibraghimov-Beskrovnaya *et al.*, 1992; Gee *et al.*, 1993; Brancaccio *et al.*, 1995). The E3 fragment of laminin-1 has been suggested to be important for kidney tubulogenesis (Klein *et al.*, 1988) and it might influence epithelial development by binding to dystroglycan (Durbeej *et al.*, 1995). Therefore it is of interest to identify intracellular molecules that bind epithelial dystroglycan.

In skeletal muscle, the dystroglycan complex binds intracellularly to dystrophin (Campbell, 1995; Ozawa *et al.*, 1995; Jung *et al.*, 1995) but dystrophin has not been detected in the kidney or in any epithelial cells. The mRNA for dystrophin is selectively expressed in muscle and in some areas of the brain (Houzelstein *et al.*, 1992). However, the dystrophin locus generates multiple transcripts coding for smaller proteins including Dp260, which is expressed in the eye (D'Souza *et al.*, 1995): Dp116, expressed in Schwann cells of the peripheral nerve (Byers *et al.*, 1993); Dp71, which is expressed in a wide variety of tissues (Schofield *et al.*,

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1994); and the recently described Dp140, which is expressed in adult brain (Lidov *et al.*, 1995). Dp71, Dp116, Dp140, and Dp260 contain the C-terminal portion of dystrophin (Blake *et al.*, 1992; Byers *et al.*, 1993; Lidov *et al.*, 1995; D'Souza *et al.*, 1995). Dystrophin binds β-dystroglycan via its second half of hinge 4 and the cystein-rich domain of the C-terminal portion (Suzuki *et al.*, 1994; Jung *et al.*, 1995). Thus, the smaller proteins should also bind β-dystroglycan. Indeed, it was recently shown that β-dystroglycan binds Dp71 and Dp260 (Jung *et al.*, 1995).

One characteristic of these smaller transcripts is that they have unique 5′ untranslated regions (Blake *et al.*, 1992; Byers *et al.*, 1993; Lidov *et al.*, 1995). This feature prompted us to design specific oligonucleotides to study the expression of Dp140 and Dp71 mRNAs by *in situ* hybridization in the developing rat kidney. We here report that both Dp140 and Dp71 mRNAs are expressed in developing rat kidney. Dp140 mRNA was selectively expressed in embryonic tubules. These findings were confirmed by immunofluorescence data obtained with monoclonal antibodies against defined epitopes of dystrophin. The epitopes of the antibodies were mapped in the current study.

MATERIALS AND METHODS

In Situ Hybridization

In situ hybridization was performed according to Durbeej *et al.* (1993) with the following modifications: hybridization was performed at 37°C instead of 42°C and washing at 60°C instead of 56°C for the Dp140 probe. When using Dp71 and dystrophin probes, washing was performed at 65°C. To detect Dp140 mRNA we used an antisense oligonucleotide probe 5′-TGT GCA AAA CCC CCT TCT GGT CCA CAC TGA TGA TTT TAG C-3′, complementary to nucleotides 23–61 of rat Dp140 first exon sequence (Lidov *et al.*, 1995). To detect Dp71 mRNA we used an antisense oligonucleotide probe 5′-GGA TTT CAC AGG CAG TCA GGG GAG GGT TAG CGC GGA GGA G-3′, complementary to nucleotides 58–97 of rat Dp71 mRNA (Blake *et al.*, 1992). A 48-mer was used to detect dystrophin 5′-CAT CCT GCA GGT CAC TGA AGA GGT TGT CTA TGT GTT GCT TTC CAA ACT-3′ complementary to nucleotides 212–260 of rat dystrophin mRNA (Nudel *et al.*, 1989). The oligonucleotide were selected with the Oligo 4.0 software (National Biosciencies Inc., Plymouth, MN). With the software, it could be confirmed that the selected oligonucleotides do not form stable dimers or hairpin loops. Furthermore, no sequence homologies to other gene products were found in the NCBI Databank. Control sections hybridized with the same amount of labeled probe plus unlabeled probe in excess did not show any specific binding.

Reverse Transcription–PCR (RT–PCR)

Poly(A)$^+$ RNA from 18-day embryonic rat kidneys was purified using a QuickPrep Micro mRNA purification kit (Pharmacia, Stockholm). RT–PCR was carried out using Superscript II RNaseH$^-$ reverse transcriptase (Gibco BRL Life Technologies, Stockholm) and *Taq* DNA polymerase (Promega, WI) according to the manufacturer's recommendations. Briefly, the reverse transcription was carried out using 1.5 μg RNA and a downstream primer directed to nucleotides 6705–6724 of mouse dystrophin mRNA (Kunkel *et al.*, 1987): 5′-TTC CCC AGT TGC ATT CAG TGT-3′. After inactivation of the reverse transcriptase for 2 min at 94°C, the 20-μl cDNA mixture was diluted by adding 50 μl water. RT controls, without the reverse transcriptase, were included and treated identically. Five microliters was added to 20-μl PCR reactions using the downstream primer used in the RT reaction and an upstream primer directed against nucleotides 1–21 of exon 1 of rat Dp140 (Lidov *et al.*, 1995): 5′-GAG GCA TTG CTG ACT GTT CTG-3′. A blank (H_2O) PCR control was also included. The PCR reaction was run for 35 cycles in a Crocodile III thermocycler (Appligene, France) with denaturation at 94°C for 1 min, annealing at 53°C for 2 min, and extention at 72°C for 1 min. The PCR products were analyzed on a 2% agarose gel.

Northern Blot Analysis

Total RNA of newborn mouse kidneys was extracted by guanidium–CsCl (Chirgwin *et al.*, 1979) from pooled deep-frozen samples. The poly(A)$^+$ containing fraction of total kidney RNA was isolated by affinity chromatography on oligo(dT)-cellulose columns. Five micrograms of poly(A)$^+$ RNA was denatured with glyoxal and electrophoresed on a 1% agarose gel. Blotting and hybridzation was performed according to Durbeej *et al.* (1995).

To detect dystrophin mRNA and mRNAs generated from the 3′end of the dystrophy locus, a 752-bp cDNA fragment was constructed from total RNA from 1-week-old mouse hearts, using reverse transcription–PCR. The oligodeoxyribonucleotides used to obtain the cDNA clone were designed using the dystrophin sequence (Kunkel *et al.*, 1987): 5′-TTG ACT GCC AAC CAC TCG GAG C-3′ (lower primer, position 11083–11104); 5′-ATT TTG CGA AGC ATC CCC GAA T-3′ (upper primer, position 10353–10374). Reverse transcription was performed with lower primer on 3 μg of RNA at 42°C in 20 μl for 1 hr using Superscript II (Gibco/BRL) reverse transcriptase. The completed reaction was diluted and used in a 40-μl standard PCR reaction including *Taq* DNA polymerase (Promega, WI). The PCR temperature profile was: 94°C 1 min, 59°C 2 min, 72°C min, for 30 cycles. The fragment was excised from a 1.5% agarose gel, purified using QIAquick gel extraction kit (Qiagen, Chatsworth, CA), and cloned in the T/A vector pCRII (In Vitrogen, San Diego, CA). The specificity of the PCR product was confirmed by restriction enzyme analysis. In addition, sequencing of about 100 bp from both ends of the insert confirmed that the clone represented dystrophin.

Identification of Monoclonal Antibody Epitopes on Dystrophin

In order to determine the dystrophin VIA4$_2$A3 antibody epitope, different GST fusion proteins containing different portions of human dystrophin (GenBank Accession No. M18533) were generated. DNA fragments encoding the last part of the spectrin-like domain (amino acid 2860 to 3000) or the cysteine-rich and C-terminal domain (amino acid 3054 to 3685) were amplified by PCR and subcloned into pGEX2TK vectors (Pharmacia). The recombinant plasmids were introduced into DH5α cells and GST fusion proteins were purified by glutathione affinity chromatography as described (Jung *et al.*, 1995). The GST–dystrophin fusion proteins were tested by Western blot analysis for reactivity with the VIA4$_2$A3 antibody.

Dystrophin epitope library in the λgt11 vector was made by digesting cDNA fragments containing the entire human dystrophin cDNA (GenBank Accession No. M18533) with DNase I in the presence of 10 m*M* $MnCl_2$. Following the addition of *Eco*RI linkers,

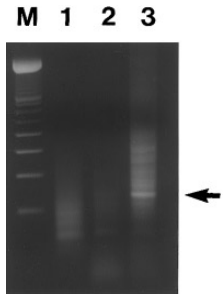

FIG. 1. Detection of Dp140 mRNA transcripts by RT–PCR. The PCR product (arrow) was generated from RNA from 18-day-old embryonic rat kidneys (lane 3). The 135-bp band can be detected neither in a control reaction where RT was omitted (lane 2) nor in a water control (lane 1). M, marker (100 bp ladder, where the lowest band represents 100 bp).

the randomly digested fragments were size-selected to <500 bp in agarose gel. These fragments were then purified, digested with *Eco*RI, and ligated into λgt11. The XIXC2 antibody was used to screen the dystrophin epitope λgt11 expression library. Positive clones were purified and phage DNA was isolated. The dystrophin cDNA fragments were excised from the phage DNA, subcloned into Bluescript vector (Stratagene), and sequenced on both strands using Sequenase II protocol (U.S. Biochemical Corp.). A dystrophin fusion protein epitope of the XIXC2 antibody was constructed by subcloning the dystrophin cDNA fragment into the *Eco*RI site of pGEX-1. The recombinant plasmid was introduced into DH5α cells and GST fusion proteins were purified by glutathione affinity chromatography as described (Jung *et al.*, 1995). The GST-dystrophin fusion protein was tested on Western blot for the reactivity with the XIXC2 antibody.

Immunofluorescence

Rat tissues were frozen in tissue Tek (Miles, Naperville, IL). Unfixed cryostat sections (7 μm) were washed in PBS, blocked in 3% BSA in PBS for 20 min, and incubated with dystrophin monoclonal antibodies VIA4$_2$A3 or XIXC2. Bound antibodies were visualized using goat anti-mouse IgM conjugated with FITC secondary antibodies. Slides were examined under a Zeiss microscope equipped with epifluorescence optics.

RESULTS

Reverse Transcriptase–PCR of Dp140 in Embryonic Rat Kidney

With the oligonucleotide primers specific for Dp140 mRNA, the expected 135-bp product was detected in the sample prepared from 18-day embryonic rat kidney (Fig. 1, lane 3). No such products were detected in either the RT–PCR control (Fig. 1, lane 2) or in the water–PCR control (Fig. 1, lane 1).

Identification of Dp140 and Dp71 mRNAs in Mouse Kidney by Northern Blot Analysis

To confirm the presence of Dp140 mRNA and Dp71 mRNA and other possible transcripts generated from the dystrophy locus in the developing kidney, we performed Northern blots using a cDNA probe directed against nucleotides 10353–11104 of dystrophin mRNA. This probe should detect mRNA for dystrophin, as well as the shorter transcripts for Dp260, Dp140, Dp116, and Dp71. As expected, the probe detected a 14-kb dystrophin transcript in 6-week-old mouse hearts (data not shown). In newborn mouse kidney, two transcripts were revealed (Fig. 2). The strong 4.7-kb band corresponds to Dp71 mRNA (Blake *et al.*, 1993) and the 7.5-kb band to Dp140 mRNA (Lidov *et al.*, 1995).

Distribution of Dp140 mRNA in the Developing Kidney

The morphological stages of nephron development (Potter, 1965; Saxén, 1987) as well as the formation of laminin-rich basement membranes in mouse and rat embryonic kidneys have been described in detail (cf. Ekblom, 1981; Ekblom *et al.*, 1990; Abrahamson, 1985). To detect Dp140 mRNA in rat embryonic kidney by *in situ* hybridization, we designed a 40-mer antisense oligonucleotide probe directed against the unique untranslated first exon of Dp140. In 15-day-old embryonic kidneys Dp140 mRNA was expressed in the comma-shaped epithelial bodies. No signals were seen in the ureter epithelium, uninduced mesenchyme, condensing mesenchyme, or in stroma (Figs. 3A and 3B). In conformity with the data in the comma-shaped stage, strong expression of Dp140 mRNA was later seen in S-shaped epithelial bodies (Figs. 3C and 3D). Higher magnifications revealed that expression was confined to the distal and proximal parts of the

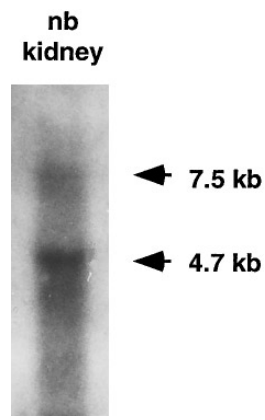

FIG. 2. Northern blot analysis of Dp140 and Dp71 mRNAs in newborn mouse kidney. 5 μg of poly(A)$^+$ RNA of newborn mouse kidneys was used. The filter was hybridized with a cDNA probe against nucleotides 10353–11104 of dystrophin mRNA. Two bands of approximately 4.7 and 7.5 kb were detected. Exposure time was 14 days.

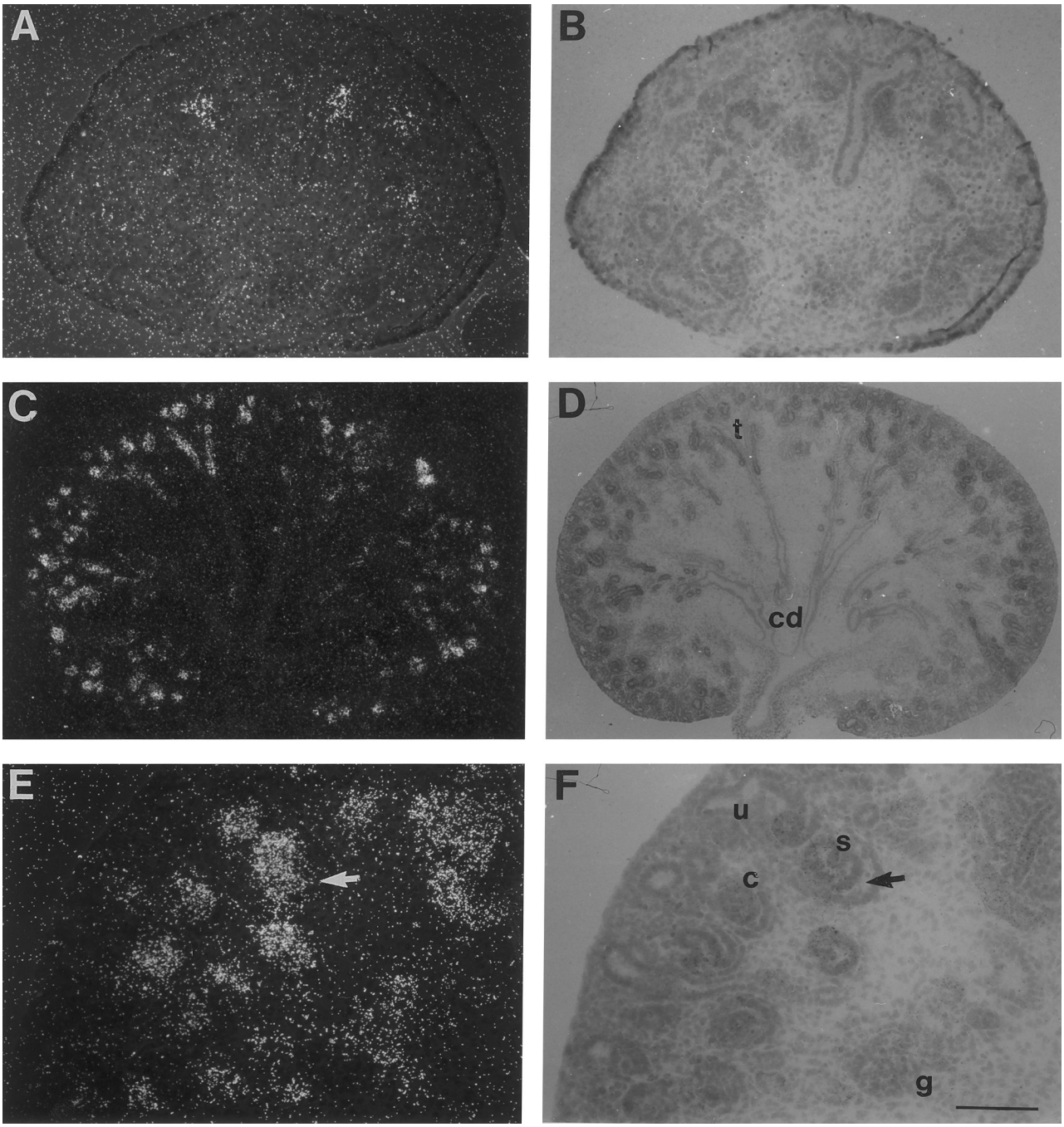

FIG. 3. *In situ* hybridization for Dp140 mRNA in the developing tubules. Sections of 15-day-old embryonic (A and B) and 18-day-old embryonic (C–F) rat kidneys were analyzed by *in situ* hybridization with an oligonucleotide probe against Dp140 mRNA. The autoradiograms are shown in darkfield (A, C, and E) and brightfield (B, D, and F). In 15-day-old embryonic kidney (A and B) signals are detected in comma-shaped bodies (c). In 18-day-old embryonic kidney (C–F) strong signals are seen in the distal and proximal part of the S-shaped bodies (s) and in the elongating straight tubules (t). Note that no signals can be detected in the ureter (u) or in the glomerular part of the S-shaped body (arrow) or in the glomeruli (g). Bar, (A and B) 80 μm; (C and D) 150 μm; (E and F) 40 μm.

S-shaped tubules, as shown here for 18-day-old embryonic kidneys (Figs. 3E and 3F). At later stages, expression of Dp140 mRNA remained in the elongating tubules that extend from the cortex and will develop into the loops of Henle. Signals continued to be absent in the epithelium of collecting ducts and in the glomeruli also at later developmental stages (data not shown). No expression of Dp140 mRNA was detected in adult rat kidney (data not shown).

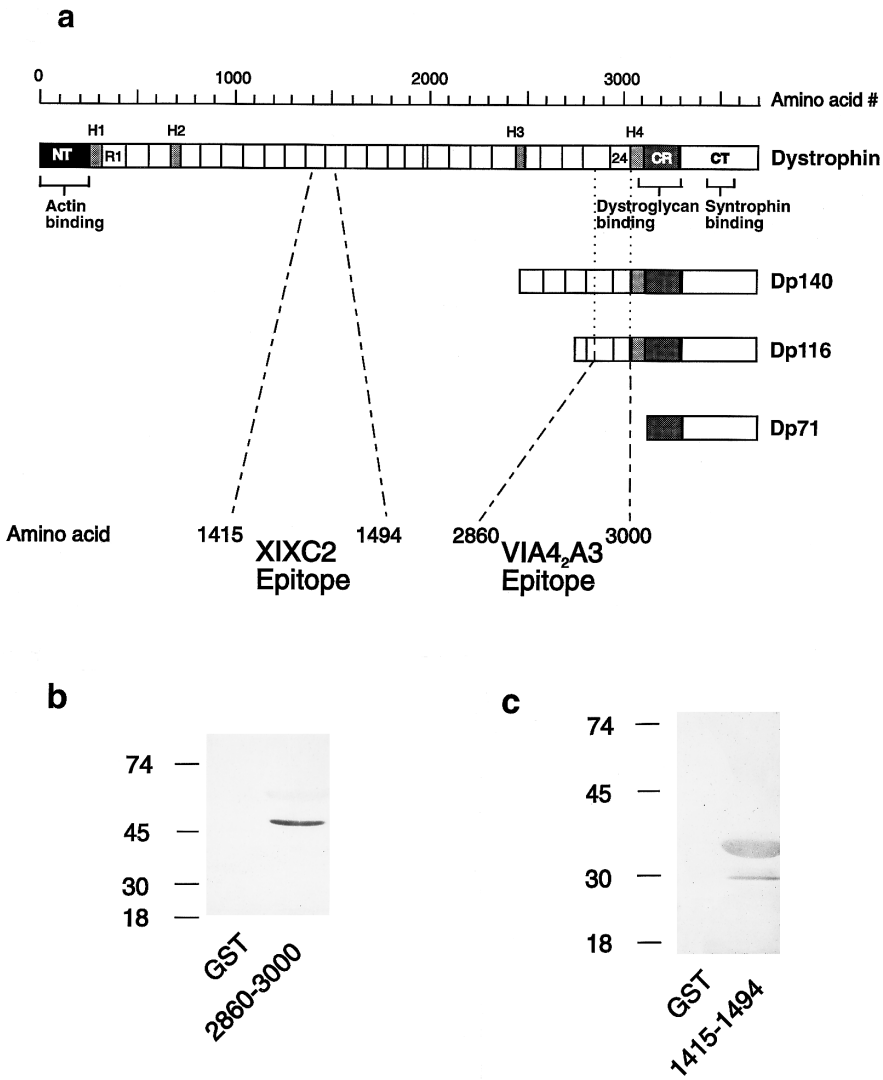

FIG. 4. Epitopes of the dystrophin monoclonal antibodies VIA4$_2$A3 and XIXC2. (a) Schematic representation of dystrophin, Dp140, Dp116, and Dp71 indicating the position of the VIA4$_2$A3 and XIXC2 dystrophin monoclonal antibodies epitopes. Amino acids were numbered based on their location in the primary structure of human dystrophin. (b and c) Immunoblots of purified glutathione *S*-transferase (GST) and glutathione *S*-transferase–dystrophin fusion proteins (containing amino acids 1415–1494 or 2860–3000) were stained with anti-dystrophin monoclonal antibody VIA4$_2$A3 (b) and XIXC2 (c). Molecular mass standards ($\times 10^3$) are indicated on the left.

Identification of the Epitope of Antibody to Dystrophin

Monoclonal antibody VIA4$_2$A3 raised against dystrophin has previously been shown to react with Dp116 in peripheral nerve (Matsumura *et al.*, 1993). Since Dp140 primary sequence contains the entire Dp116 amino acid sequence (Fig. 4a), the VIA4$_2$A3 antibody should also react with Dp140. To determine the location of VIA4$_2$A3 antibody epitope, GST–dystrophin fusion proteins containing either the last part of the spectrin-like domain (amino acid 2860 to 3000) or the cysteine-rich and C-terminal domain (amino acid 3054 to 3685) were prepared. The VIA4$_2$A3 antibody reacted with the fusion protein containing amino acid 2860 to 3000 (Fig. 4b) but not with the fusion protein containing amino acids 3054 to 3685 (data not shown). Therefore VIA4$_2$A3 antibody epitope is located within residues 2860 to 3000 of dystrophin also contained in Dp140 and Dp116 but not in Dp71 (Fig. 4a).

Detection of Dp 140 by Immunofluorescence in the Developing Kidney

Immunostaining with antibody VIA4$_2$A3 was detected both in distal and proximal parts of the comma and S-shaped tubules, but not in the glomerular part, as shown here for 18-day-old

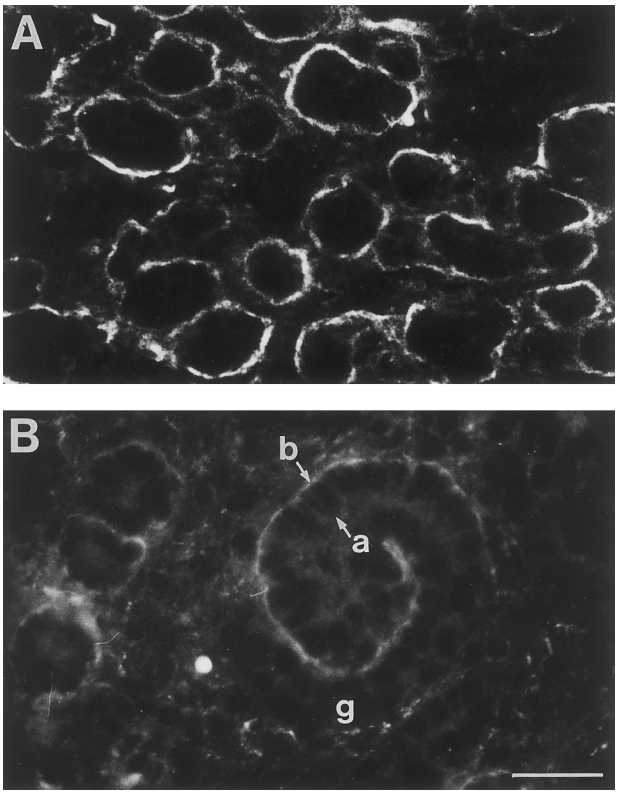

FIG. 5. Immunolocalization of dystrophin in embryonic skeletal muscle and Dp140 in embryonic kidney. Sections of 18-day-old embryonic skeletal muscle (A) and kidney (B) were stained with VIA4$_2$A3 antibodies and detected with indirect immunofluorescence. In skeletal muscle dystrophin is localized to the sarcolemmal membrane. The antibody detects Dp140 in embryonic kidney and the staining is confined to the basement membrane region (b) of the distal and proximal parts of the comma-shaped body. Very little staining, if any, is seen on the apical side (a) of the epithelial cell. Note that no staining can be seen in the basement membrane region of the glomerular part (g). Bar, 42 μm.

embryonic kidneys (Fig. 5B). The staining was confined to the basal side of cells close to the basement membrane region (Fig. 5B). Occasionally some staining was seen also on the apical side. As the tubules elongated, staining was noted in the basement membrane region of elongating tubules but not in the collecting ducts or glomeruli (data not shown). In adult kidney, no staining was detected (data not shown). Antibody VIA4$_2$A3 also reacted with dystrophin in the sarcolemmal membrane of embryonic skeletal muscle (Fig. 5A). Sections of 18-day-old embryonic kidneys were stained with the monoclonal antibody XIXC2 that detects dystrophin, but neither Dp140, Dp116, nor Dp71 (Fig. 4a). As expected, antibody XIXC2 reacted with the sarcolemmal membrane of embryonic skeletal muscle but no staining was seen in embryonic kidney (data not shown).

Localization of Dp140 mRNA in Other Parts of the Rat Embryo

To examine whether Dp140 mRNA was expressed in epithelium of other organs, we performed *in situ* hybridization on sections of 15-day-old whole rat embryos. However, no expression of Dp140 mRNA was seen in any other epithelia. Instead, expression was noted in dorsal root ganglia (Figs. 6A and 6B) and in the mantle layer of the spinal cord (Figs. 6C and 6D).

Expression of Dp71 mRNA and Dystrophin mRNA in Embryonic Kidney

An antisense oligonucleotide probe directed against the unique 5′ untranslated region of Dp71 mRNA was used in the *in situ* hybridization experiments. This probe cannot distinguish between full-length Dp71 (apodystrophin-1) and its 3′ truncated version (apodystrophin-3; Tinsley *et al.*, 1993). To detect dystrophin mRNA, an oligonucleotide probe against the 5′ region of full-length dystrophin mRNA was designed.

To verify that the probes were specific for each of the mRNA, we first performed *in situ* hybridization on whole 15-day-old embryonic rat embryos. As expected, the oligonucleotide against dystrophin reacted only with tissues previously shown to express dystrophin mRNA (Houzelstein *et al.*, 1992); cardiac muscle (Figs. 7A and 7B), skeletal muscle, smooth muscle, and also some restricted areas in the brain (data not shown). *In situ* hybridization with the oligonucleotide specific for Dp71 also matched previous findings for expression of Dp71 mRNA (Schofield *et al.*, 1994); no reaction was seen in cardiac muscle. However, a faint expression of Dp71 mRNA was seen in the epicardium and in the cells in the outflow tract probably representing smooth muscle or endothelial cells (Figs. 7C and 7D). Also, strong expression of Dp71 mRNA was seen in the developing eye; in the inner neural layer of the optic cup and in the anterior wall of the lens vesicle (Figs. 8A and 8B). Among other locations, Dp71 mRNA was expressed in the liver and in the CNS (data not shown).

Expression of Dp71 mRNA has been previously noted in the embryonic mouse kidney, but the cell types expressing it were not identified in detail (Schofield *et al.*, 1994). In 18-day-old embryonic rat kidneys, a faint expression was seen in the ureter bud and the condensing mesenchyme. A stronger expression of Dp71 mRNA was seen in the developing tubules. Neither the uninduced mesenchyme nor the developing stroma expressed Dp71 mRNA. In addition, some expression was also noted in glomeruli, elongating tubules, and collecting ducts (Figs. 9A and 9B). In contrast, no dystrophin mRNA was detected in 18-day-old embryonic kidneys (Figs. 9C and 9D).

DISCUSSION

Dystroglycan is a recently described novel receptor complex for basement membranes in muscle and epithelia (Campbell, 1995; Durbeej *et al.*, 1995). Laminins are major glycoproteins of basement membranes and they bind dystroglycan with high affinity (Sunada and Campbell, 1996). It therefore becomes important to compare the role of dystro-

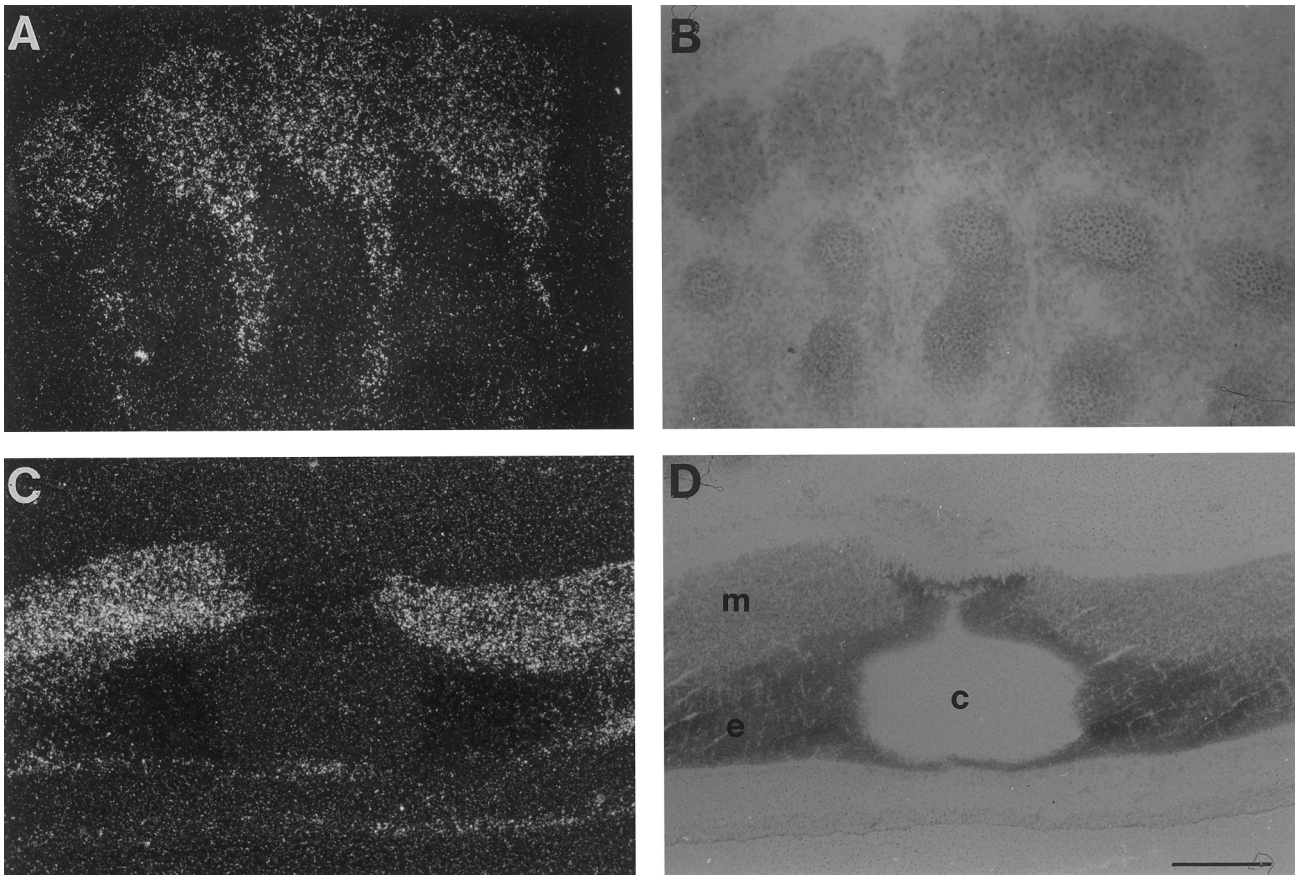

FIG. 6. *In situ* hybridization for Dp140 mRNA in the rat embryo. Parasagittal sections of 15-day-old rat embryos were hybridized with a synthetic oligonucleotide probe against Dp140 mRNA. A and C are shown in darkfield and B and D are shown in brightfield. Dp140 mRNA is expressed in the dorsal root ganglia (A and B) and in the mantle layer of the spinal cord (C and D). m, mantle layer; e, ependymal layer; c, central canal. Bar, 500 μm.

glycan and integrins for muscle and epithelial morphogenesis, and to identify intracellular proteins which bind to the different receptors. The 427-kDa dystrophin which in muscle binds to dystroglycan is not expressed by epithelial cells (Houzelstein *et al.*, 1992) and the intracellular proteins which bind epithelial dystroglycan thus remain to be identified. Here we show that some epithelial cells in developing kidney express Dp140, a transcript from the dystrophin locus. Moreover, epithelial cells both in the kidney and elsewhere express Dp71. The dystrophin locus encodes multiple transcripts coding for smaller proteins including Dp71, Dp116, Dp140, and Dp260 which all share the carboxyterminus with dystrophin (Blake *et al.*, 1992; Byers *et al.*, 1993; D'Souza *et al.*, 1995; Lidov *et al.*, 1995). The β-dystroglycan binding site has been localized to the second half of hinge 4 and the cystein-rich domain of dystrophin (Suzuki *et al.*, 1994; Jung *et al.*, 1995). The short dystrophin variants expressed in embryonic kidney tubules contain these sites and should thus bind dystroglycan. Proteins from brain with the size of Dp71 and Dp260 bind to β-dystroglycan (Jung *et al.*, 1995). Taken together, current results raise the possibility that Dp140 and Dp71 during kidney tubulogenesis bind to β-dystroglycan. It will be important to test whether Dp140 also binds dystrophin associated proteins in a similar manner as Dp71 and Dp260.

In order to detect the cell types which express Dp140 and Dp71 mRNA, we generated specific oligonucleotides for their unique 5′-untranslated regions and used them by *in situ* hybridization. Expression of Dp140 mRNA was strictly confined to the developing distal and proximal tubular cells. The mesenchyme did not express Dp140, but epithelial cells both in the collecting ducts and glomeruli were also devoid of expression. Immunostaining with antibody VIA4$_2$A3 confirmed the mRNA findings. In the present study we mapped the epitope of the VIA4$_2$A3 dystrophin antibody with the use of recombinant fusion proteins. The antibody VIA4$_2$A3 reacted with a dystrophin fusion protein containing amino acids 2860 to 3000 and therefore the epitope is located within these residues of dystrophin also found in Dp140 and Dp116. Since neither dystrophin nor Dp116 mRNA (Houzelstein *et al.*, 1992; Byers *et al.*, 1993) are expressed in the kidney, the current immunostainings should represent

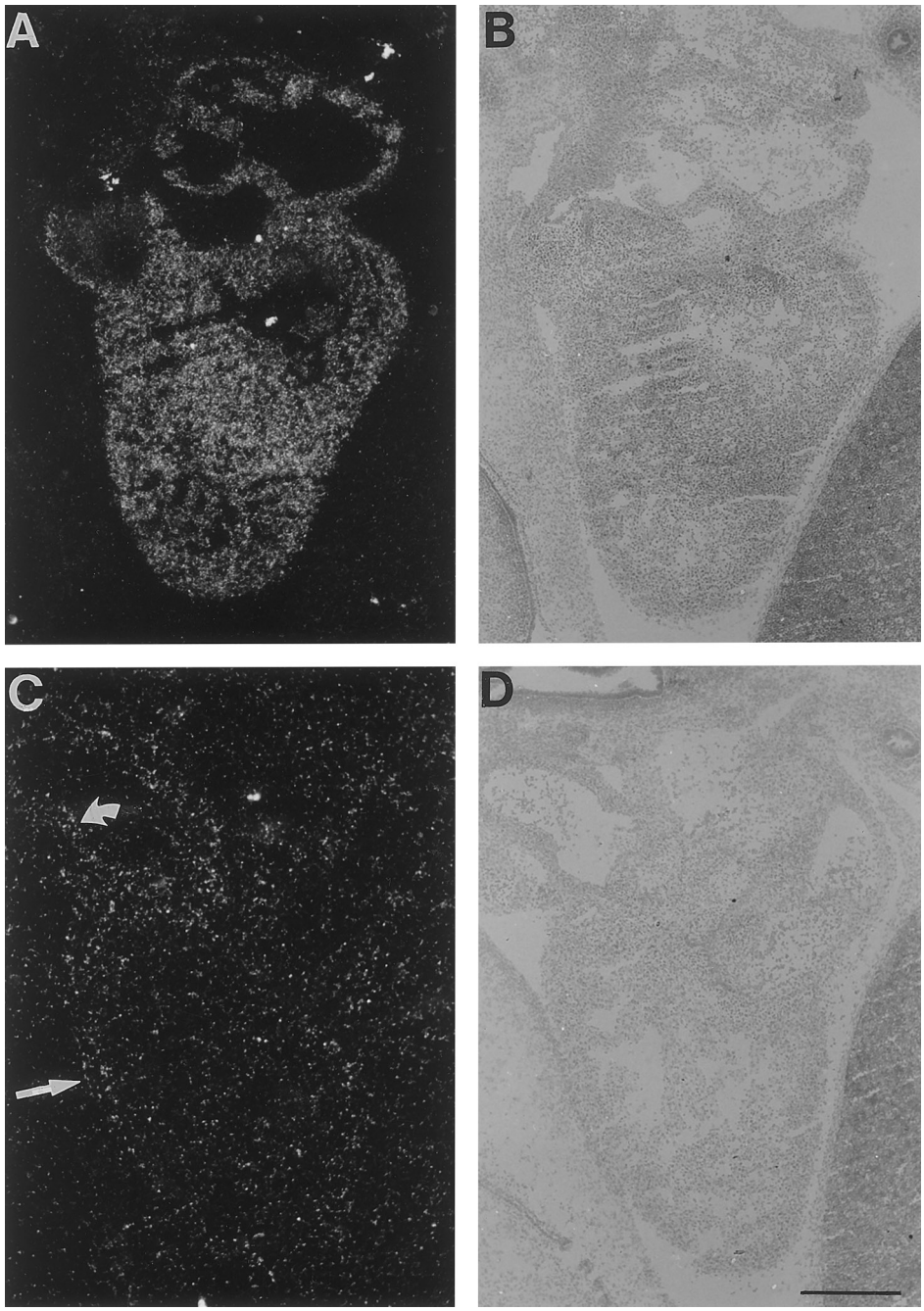

FIG. 7. *In situ* hybridization for dystrophin mRNA and Dp71 mRNA in the embryonic rat heart. *In situ* hybridization for dystrophin mRNA (A and B) and for Dp71 mRNA (C and D) was performed on sections of 15-day-old embryonic rat hearts. Dystrophin mRNA is expressed at a high level in the cardiac muscle, whereas Dp71 mRNA is not. However, a faint expression of Dp71 mRNA is seen in the epicardium (thin arrow) and in the cells of the outflow tract (fat arrow). Bar, 250 μm.

Dp140 protein. In conformity with the mRNA data, VIA4$_2$A3 antibody selectively bound to the embryonic distal and proximal tubules. Moreover, antibody VIA4$_2$A3 detected an antigen intracellularly close to the basal cell membranes. The basal location is compatible with its assumed function as an intracellular binding protein for dystroglycan.

The presence of Dp140 mRNA in embryonic kidney was verified by RT–PCR and by Northern blot analysis. Northern analysis of newborn whole kidneys suggested a stronger

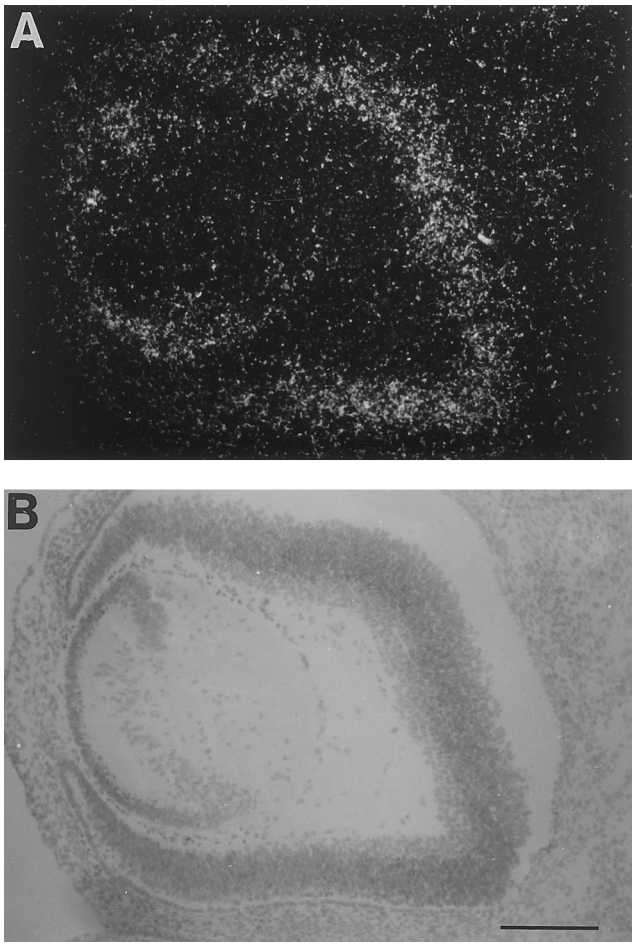

FIG. 8. *In situ* hybridization for Dp71 mRNA expression in the developing rat eye. Autoradiograms are shown in darkfield (A) and brightfield (B). In 15-day-old embryonic rat eye, the expression of Dp71 mRNA is restricted to the inner neural layer of the optic cup and to the anterior wall of the lens vesicle. Bar, 200 μm.

overall expression of Dp71 than Dp140 in kidney of newborn mice. Dp 140 may nevertheless be more abundant than Dp71 in the embryonic tubules because Dp71 is more widely expressed. In contrast to the mRNA for Dp140, the mRNA for Dp71 was expressed by all epithelial cells and also by the condensing mesenchyme. In the tubules the *in situ* hybridization signals for Dp71 appeared to be weaker than those of Dp140. The uninduced mesenchyme and the stroma contained very little if any mRNA for Dp71. Another difference was that Dp140 mRNA expression was confined to the CNS and kidney, whereas some Dp71 mRNA was expressed in a wide variety of tissues including kidney, CNS, liver, and eye.

The transmembrane 43-kDa β-dystroglycan and extracellular 156-kDa α-dystroglycan are encoded by a single mRNA (Ibraghimov-Beskrovnaya *et al.*, 1992). In the embryonic kidney this transcript is specific for epithelial cells (Durbeej *et al.*, 1995). The expression of Dp 140 matches the expression of dystroglycan in a subset of embryonic kidney epithelial cells, the developing tubular cells. These tubules express laminin α1 chain but very little laminin α5 chain (Ekblom *et al.*, 1990; Durbeej *et al.*, 1996). It is thus tempting to speculate that α-dystroglycan in these cells binds to laminin α1 chain, and β-dystroglycan to Dp140. Since Dp140 mRNA is not expressed in the ureter or in the epithelium of glomeruli, other intracellular dystrophin-like proteins binding to β-dystroglycan should exist in these cells. Utrophin, a homolog of dystrophin, binds to the dystroglycan complex (Matsumura *et al.*, 1992) and is found in focal adhesions in many cell types (Belkin and Burridge, 1995). Utrophin is expressed in the embryonic kidney (Schofield *et al.*, 1994) and could thus together with Dp140 and Dp71 act as a dystroglycan-binding protein in developing kidney tubules. It was recently shown that exogenously added Dp71 could restore the normal levels of dystrophin-associated proteins in muscle in transgenic mdx mice that do not have dystrophin. The data suggest that Dp71 is capable of interacting with the dystrophin-associated proteins in a manner similar to dystrophin. Nevertheless, the presence of Dp71 was not sufficient to alleviate symptoms of muscle degradation (Ohlendieck and Campbell, 1991; Cox *et al.*, 1994; Greenberg *et al.*, 1994), suggesting different functions for the dystrophin variants. It is possible that Dp140 and Dp71 are involved in embryonic development of epithelial cells, although there are genetic studies showing that low levels of some of the dystrophin transcripts do not lead to defects in epithelial cells. The mdx^{3Cv} mouse cell line, which produces very little or no Dp71, might be a model system to study the role of Dp71 and Dp 140 in epithelial development. No kidney or epithelial defects have yet been reported in these mice (Cox *et al.*, 1993). In humans, there are some reports of mutations in the C-terminal portion of dystrophin that cause Duchenne muscular dystrophy; expression of dystrophin and Dp71 is low in these individuals (Towbin *et al.*, 1989; Roberts *et al.*, 1992). However, no kidney defects have been reported in these individuals. It will important to study kidney development and the expression levels of Dp 71 and Dp 140 all in such cases more thoroughly.

Although Dp140 and Dp71 should bind to dystroglycan in a similar fashion in epithelium as dystrophin to dystroglycan in muscle, these smaller proteins do not contain the N-terminal portions of dystrophin which bind to F-actin. It thus remains unclear whether dystroglycan and the cytoskeleton are connected in epithelial cells. Yang *et al.* (1995) recently demonstrated a direct association between β-dystroglycan and Grb-2, indicating that the dystroglycan complex might also be involved in signal transduction. Dystroglycan-dependent signal transduction may occur during epithelial morphogenesis of the kidney (Durbeej *et al.*, 1995) and our current data suggest that Dp71 or Dp140 could be involved in such processes.

In summary, we have provided evidence that two shorter transcripts from the dystrophin locus, Dp140 and Dp71, are expressed in epithelial cells in the developing kidney, and that Dp71 in addition is more widely expressed. It might

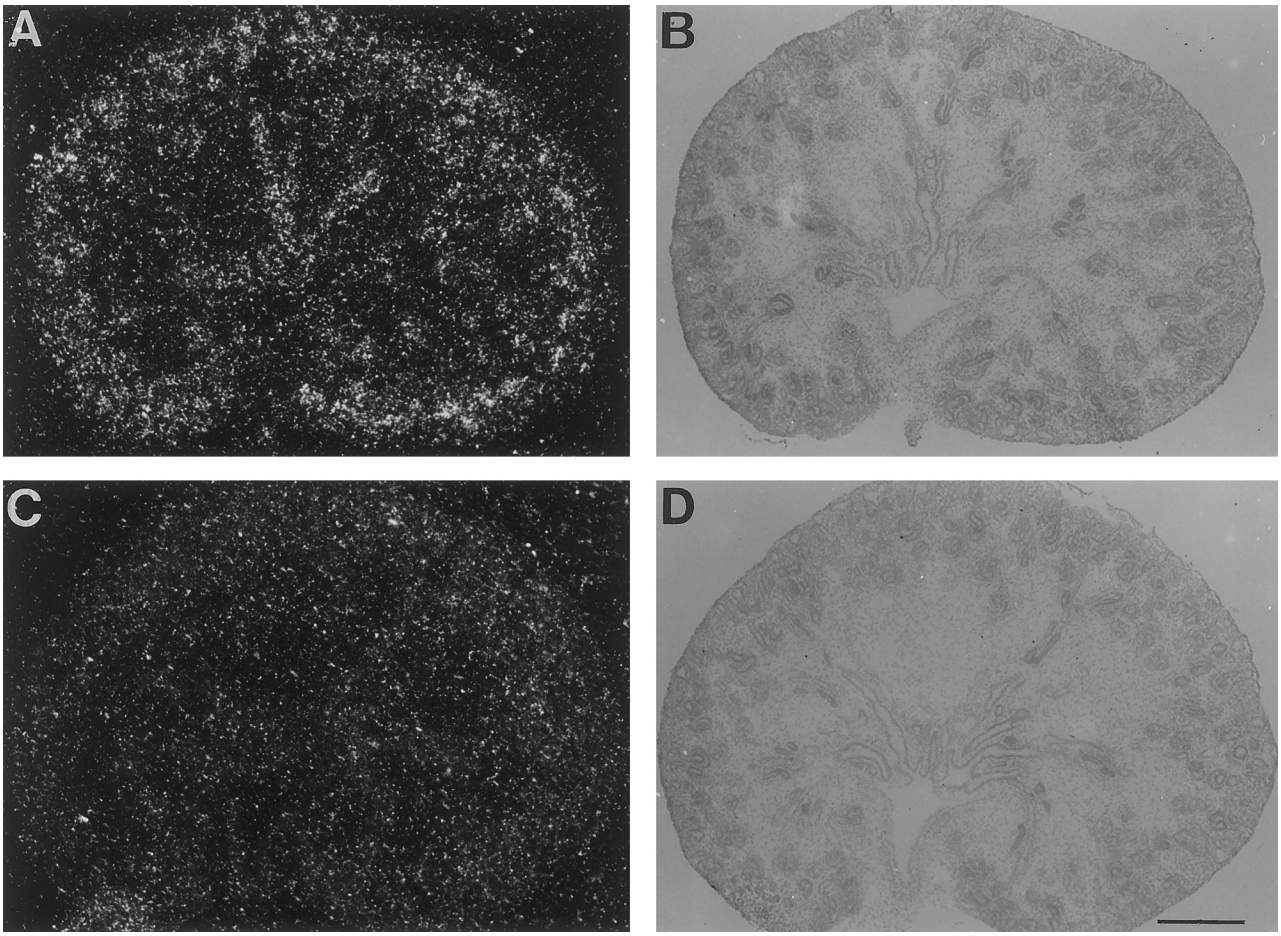

FIG. 9. *In situ* hybridization for Dp71 mRNA and dystrophin mRNA expression in the developing rat kidney. Sections of 18-day-old embryonic rat kidneys were analyzed by *in situ* hybridization with an oligonucleotide probe against Dp71 mRNA (A and B) and dystrophin mRNA (C and D). The autoradiograms are shown in darkfield (A and C) and brightfield (B and D). Expression of Dp71 mRNA is seen all epithelial cells and also in the condensing mesenchyme. Note that the uninduced mesenchyme and the stroma contain very little, if any, Dp71 mRNA. Note complete lack of dystrophin mRNA in 18-day-old embryonic rat kidneys. Bar, 150 μm.

be of interest to identify the molecules in the kidney that bind Dp71 and Dp140 and to study whether these protein complexes affect epithelial cell development.

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